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ON THE INFLUENCE OF STAPHYLOCOCCUS TOXINS ON CELL CULTURES FROM MONKEY KIDNEYS

[Following is translation of a German-language article by M. Kienitz and G. Schmelter, Children's Clinic of Münster University, in Zentralblatt für Bakteriologie und Parasitologie (Central Journal for Bacteriology and Parasitology), Vol. 193, 1964, No. 4, pp. 447-453.]

SUMMARY: In the continuation of long-term studies on the mechanism of action of Staphylococcus toxins, a report is made on the influence of these substances on monkey kidney cell cultures. Crude filtrates of the laboratory strain SG 511 led to extensive break-up of the cell formations and change in form of the individual cells. A filtrate of the strain S-6 induced similar changes, while the Staphylococcus toxin of the Behring firm caused other kinds of morphological reactions. Irrespective of their relatively high content of α-toxin, filtrates from the Wood 46 strain did not adversely affect either the growth or the structure of the cultures. Neutralization tests showed that the damaging effects to the cells that were observed are in no way related to the tested haemolyzing toxins. In the experimental lay-out used, the Staphylococcus enterotoxin possesses no cytopathogenic or cytotoxic action. The authors regard two as yet not precisely defined products of metabolism responsible for the damage to the cells. Well-founded evidence concerning the identity of these substances with already known toxins (e.g. lethal toxin) is not yet to hand. (Original English text of source.)

Introduction

During long-continued investigations on the mechanism of action of various staphylococcus toxins, the question arose for a possible specific effect of these substances on cell cultures. The surprisingly few communications presently available on this subject (cf. note in annex) frequently contain contradictions which we attempted to clarify through our own experiments. Los Fargues and Delaunay (1946) observed,

after action for 60 min by staphylococcus toxin (an exact definition of the toxin is absent from the communication) on cultures of spleen tissue, rapid decay of the cells with earlier signs of degeneration. If the toxin was heated to 100° C or neutralized by respective antisera, the result of experimentation corresponded to that of the control series with non-toxinic media. Less impressive were the changes noted by Thal and Egner (1961) in cultures of human esophageal epithelium and/or fibroblasts of the rabbit after addition of a toxin derived from the strain Wood-46. For terminal decay of the cells, an interval of action of four hours was shown to be required and the first symptoms of damage in the form of vacuolization and granulation of the cells occurred after two hours. Special interest was aroused by the report of Guerin et al (1961) on characteristic and easily distinguished effects of the hemolizing toxins (α-, β- and δ-toxin) and of the enterotoxin. Unfortunately, here also the necessary technical data are absent because this is a still unpublished lecture, according to a communication by Jackson (1963).

Materials and Methodology

Staphylococcus toxins: The crude toxins of the strains Wood-46 (obtained from "Human-Institute" Budapest), SG-511 (obtained from Institute of Hygiene of Münster University) and S-6 (obtained from Food Research Institute in Chicago) were prepared by a method described earlier in detail (Kienitz, 1962) and tested immediately prior to begin of experimentation for their content of α-toxin, β-toxin and enterotoxin. In addition, we utilized the commercial staphylococcus toxin of the "Behringwerke" (Op-No. 9659; LH = 0.09). For the evaluation of enterotoxin effects important in practice, we utilized enterotoxin preparations, partially purified in our own laboratory, of the strains S-6 and 1283 as well as a highly purified enterotoxin of the strain S-6 of the Food Research Institute at Chicago (we are obligated to Dr. M. S. Bergdoll for making the substance available to us) which regularly caused vomiting in Rhesus monkeys already at a dose of 10 γ (Bergdoll, 1962). All three enterotoxins were available in lyophilized form and were utilized in diluation with physiological salt solution. For dosage, cf. table 2.

Antitoxins: For neutralization tests, we utilized both the staphylococcus serum of the "Behringwerke" (400 AE/ml diluted 1 : 20) as well as the standard α-antitoxin (20 AE/ml) of the "Statens Seruminstitute" at Copenhagen. The toxin-antitoxin mixtures were adjusted by means of the hemolysis test (erythrocytes of rabbit and sheep). To the serum quantities producing neutralization of the toxin in each case, we further added as a "safety factor" 25% of the required dose. Monkey-kidney cell cultures: Trypsinized cell suspensions in a dilution 1 : 50 (Behringwerke) were further diluted with a mixture of Hanks solution and TCM nutrient solution in equal parts and with the addition of 5% calf serum at a ratio 1 : 10, and transferred to test tubes after the respective control for sterility. The cell density was about 80,000/ml. After incubation for

4-5 days in the closed tube at 57° C and a check on cell growth, we changed the nutrient medium.

Experimental arrangement: On the morning of each day of experimentation, we transferred toxins and non-toxinic media to the cell cultures in increasing amounts and always in two series. Readings were made with the so-called "chemist microscope" after 12, 24, 48, 72 and 96 hours. In some batches, we changed the medium 2 hours after the begin of the experiment and simultaneously removed the greater part of the toxins added. The findings were generally evaluated by two investigators and reported in two separate protocols. This produced an extensive concordance of the findings which was identified with ∅, +/-, +, ++ and +++, depending on the degree of cellular change. During the experiment, we withdrew 30 specimens for control of sterility and none of these showed any germ growth among 200 batches (= 400 cultures). We found only twice an impurity of the culture (demonstration of B. subtilis) only twice which was recognized already microscopically due to the turbidity of the content of the test tube.

Findings

1. Influence of Crude Filtrates on Monkey-Kidney Cell Cultures

Under identical conditions, we investigated the influence of the crude filtrates Wood-46, SG-511 and S-6 as well as the staphylococcus toxin of the Behringwerke on the cell cultures. At a dose of 0.1 ml, the filtrate Wood-46 (α -toxin titer 256, no β-toxin) produced a just notable decomposition of the cell clusters. The addition of 0.2 ml toxin resulted only in somewhat more pronounced change of structure. When increasing the filtrate added to 1.5 ml, no change of the cell picture could be recognized. Filtrate SG-511 (α-toxin titer 16, no β-toxin) completely destroyed, after 2 hours, the cell clusters while simultaneously transforming the individual cell into a filiform structure. Produced by 0.1 ml of filtrate, this effect could be demonstrated in attenuated form also with the filtrate S-6 but the dose had to be increased to 0.2 ml of filtrate for this result. The dead cells appeared as filiform and as spherical structures. At a dose of 0.2 ml, the staphylococcus toxin Behringwerke caused a massive destruction of the cell clusters showing a uniform characteristic of spherical deformation of the individual cell. Similar appearances are found after inoculating cell cultures with poliomyelitis virus.

2. Toxin-Antitoxin Tests

The action described above of the filtrates Wood-46, SG-511 and S-6 could be neither neutralized nor even attenuated by the use of toxin-antitoxin mixtures. After heating to 100° C for 30 min, the filtrates as well as the non-toxinic nutrient medium did not influence the growth and structure of the cultures in any manner. Noteworthy for

Table 1

Neutralization tests with filtrate SG-511 and S-6. Each result corresponds to the mean from not less than 5 batches into identical tests.

(a) Toxinbezeichnung	(b) Dosis (ml)	(c) Ablesungsergebnisse nach 12	24	48	72	96 Std. (o)
Filtrat SG 511 (d) + NaCl-Lösung (e)	0,2 + 0,2	+++	+++	+++	+++	+++
Filtrat SG 511 + ATα	0,2 + 0,2	+++	+++	+++	+++	+++
Filtrat SG 511 + ATB	0,2 + 0,2	+++	+++	+++	+++	+++
Filtrat S-6 + NaCl-Lösung (e)	0,2 + 0,2	++	+++	+++	+++	+++
Filtrat S-6 + ATα	0,2 + 0,2	++	+++	+++	+++	+++
Filtrat S-6 + ATB	0,2 + 0,2	++	+++	+++	+++	+++
Behring-Toxin + NaCl-Lösung	0,25 + 0,25	++	+++	+++	+++	+++
Behring-Toxin + ATα	0,25 + 0,25	+−	++	++	+++	+++
Behring-Toxin + ATB	0,25 + 0,25	+−	+	+	+	++
ATB + NaCl	0,25 + 0,25	0	0	+−	+−	+−

Zeichenerklärung: (f)
ATα = α-Antitoxin Statens Seruminstitut Copenhagen; (g)
ATB = Staphylokokkenserum Behringwerke (h)
ø = Kultur unverändert; (i)
+− = Fragliche Auflockerung des Gewebes (k)
+ = Auflockerung der Struktur und Ablösung einzelner Zellen; (l)
++ = Deutliche Zerstörung der Zellverbände und Verformung der Einzelzelle; (m)
+++ = Vollständige Zerstörung der Struktur mit Verformung aller sichtbaren Zellen. (n)

Legend:

(a) - toxin designation;
(b) - dose (ml)
(c) - readings after...;
(d) - filtrate;
(e) - NaCl solution;
(f) - explanation of symbols;
(g) - α-antitoxin from Statens Seruminstitute Copenhagen;
(h) - staphylococcus serum Behringwerke;
(i) - culture unchanged;
(k) - doubtful loosening of tissue;
(l) - loosening of structure and separation of individual cells;
(m) - perceptible destruction of cell clusters and deformation of the individual cell;
(n) - complete destruction of structure with deformation of all visible cells;
(o) - hrs.

us was only the extensive reduction of the cell-damaging effect by the staphylococcus toxin Behringwerke through the homologous antiserum. Minor manifestations of degeneration were produced in a similar manner also by the serum alone so that the separation of individual cells and minor loosening of the cell clusters observed may be ascribed more readily to the conservation medium (0.5% phenol). Toxin heated to 100° C had no influence on the tissue culture. Details are shown in Table 1.

3. The Action of Staphylococcus Enterotoxin on Cell Cultures

The findings of the tests with filtrate S-6 permitted the assumption of cell-damaging effect of the enterotoxin. As will be seen from table 2, however, this component is lost with increasing purification of the substance. Changes in the structure of the cell cluster, still recognizable after the addition of partially purified enterotoxin (S-6 and 1283), could no longer be demonstrated with the optimum purified and highly active preparation S-6.

Table 2

Experiments on the influence of enterotoxin derived from strain S-6 on monkey-kidney cell cultures.

(a) Toxinbezeichnung	(b) Dosis (ml)	(c) Ablesungsergebnis nach 12	24	48	72	96 Std. (l)
Rohfiltrat S-6 (d)	0,05			+ –	+ –	+ –
	0,2			+ –	+ –	+
	0,2	–	– +	+ + +	+ + +	+ + +
	0,4	+ –	+ + +	+ – +	+ + +	+ + +
Partiell gereinigtes (e) Enterotoxin S-6 (250 γ/ml)	0,1					
	0,2				+ –	+ –
	0,3		+ –		+ –	+ –
	0,6	+ –	+ –			+ +
	0,8	+	+	+ +	– +	+ +
Optimal gereinigtes (f) Enterotoxin S-6 (180 γ/ml)	0,1					
	0,2					
	0,4					
	0,6					
	0,7					+ + + [1]
	0,9					
NaCl-Kontrolle (g)	0,2					
	0,4					
	0,8					
Unbeimpfte Kontrollen (h) (10 Röhrchen)	–					

[1] Röhrcheninhalt getrübt; Kultur: Bac. subtilis. (i)
Zeichenerklärung siehe Tabelle 1. (k)

Legend:

(a) - toxin designation;
(b) - dose (ml);
(c) - reading after...;
(d) - crude filtrate S-6;
(e) - partially purified enterotoxin S-6;

Table 2 Legend (continued):

(f) - optimum purified enterotoxin S-6;
(g) - NaCl control;
(h) - non-inoculated control (10 tubes);
(i) - turbid tube content; B. subtilis culture;
(k) - explanation of symbols, cf. table 1;
(l) - hrs.

Discussion of Findings

If we base ourselves on the accuracy of our experimental arrangement here as an answer to the question as we stated it, we can then note that a specific effect of the known staphylococcus toxins on cell cultures probably does not exist. For α-toxin as well as for enterotoxin, we can adequately demonstrate this statement; we can not yet entirely negate such an effect for β-toxin. Consequently, the findings here presented considerably differ from those of Guerin et al (1961) so that the question remains open whether fibroblast cultures, in contrast to cultures from monkey-kidney cells, are actually able to react with respectively specific changes of form under the action of staphylococcus toxins. Our present experience speaks against this assumption; however, only a systematic reproduction of the experiments of Guerin et al can justify a definite conclusion.

The postulate of a heat-labile component which can not be detoxicated by α-antitoxin and develops cytopathogenic and/or cytotoxic properties on monkey-kidney cells, can be based primarily on the tests made with filtrate SG-511. It further seems reasonable, in view of the two different, morphologically easily distinguishable cell pictures (cf. tests with filtrate SG-511 and/or S-6 and staphylococcus toxin Behringwerke), to assume the existence of a second substance, such as exists in the staphylococcus toxin Behringwerke, which is extensively neutralized in regard to its cell-damaging effect after addition of the corresponding antiserum. Earlier experiments on white rats (Kienitz et al, 1961) showed that the lethal effect of the staphylococcus toxin Behringwerke is eliminated in the presence of the staphylococcus serum Behringwerke but not by the addition of sufficient (i.e. adequate) amounts of pure α-antitoxin. The parallel between the findings from animal experimentation and tissue cultures probably is not accidental but still lacks definite proof. We should also recall that the laboratory strain SG-511, in the experimental staphylococcus sepsis of the white rat, also definitely did not show the expected non-virulence but caused definite organic changes (Kienitz et al, 1960). There thus results a certain correlation with the virulence problem whose solution has been approached only to a very minor extent. We trust that future investigations will produce more detailed clarification of the character and mechanism of action of these metabolic products of a number of staphylococcus strains. At the present time,

we are only certain that monkey-kidney cell cultures are not suitable for the differentiation of staphylococcus toxins and specifically for the demonstration of staphylococcus enterotoxin.

Annex

Authors' note (added after proofreading of original communication): The ineffectiveness of staphylococcus enterotoxin (serological types A and B) on various types of cell cultures was confirmed by Sugiyama (personal communication 1964). Milone (98th Annual Meeting of American Public Health Association 1961, Detroit) came to the same conclusion, after checking the findings of Guerin et al (1961), as we that the cellular changes obtained with crude filtrates of enterotoxin-forming staphylococcus strains can no longer be demonstrated with increasing purification of the preparations. The investigations of English and American authors of which we have become aware only now, on the effect of α-toxin on cell cultures from human and animal substance can not be correlated with our experiments. In addition to Nogrady and Burton (Pathology and Biology, vol. 9, p. 831, 1961), Lawrence (British Journal of Experimental Pathology, vol. 40, p. 8, 1959) as well as Gablika and Solotorovsky (Journal for Immunology, Vol. 88, p. 505, 1962), Artenstein et al (Yale Journal of Biological Medicine, vol. 35, p. 373, 1963) and/or Morton et al (idem, vol. 35, p. 382, 1963) furnished impressive demonstrations for the cell-destroying action of α-toxin where the cell membrane is regarded as the direct point of attack. Although the authors quoted did not utilize monkey-kidney cell cultures and worked in part with highly purified toxin preparations, the discrepancy with our findings is provisionally not clarified. Within the purport of our further investigations, we shall therefore give particular attention to α-toxin and include purified toxin in our experimentation. If hemolytic activity, lethal action and dermonecrotic effect actually are properties of only one toxin -- recently published communications of American authors speak for this thesis -- we would then have to consider, in neutralization experiments with antitoxin, in addition to neutralization of hemolysis also the inhibition of other factors. Obviously, in that case, a great part of our findings (cf. tests with crude filtrate Wood-46 and SG-511) then remains incomprehensible so that we are not in a position, even after becoming cognizant of the work referred to above, to change the viewpoints presently held.

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